negative agglutination seen in patients with rheumatoid arthritis.

1. Singer, J. M., Plotz, C. M., *Am. J. Med.*, 1956, v21, 888.
2. Ziff, M., Brown, P., Badin, J., McEwen, C., *Bull. Rheumatic Dis.*, 1954, v5, 75.
3. ———, *Am. J. Med.*, 1956, v20, 500.
4. Hall, A. P., Mednis, A. D., Bayles, T. B., *N. Eng. J. Med.*, 1958, v258, 731.
5. Olsen, C. R., Rantz, L. A., *Arthritis and Rheumatism*, 1958, v1, 54.
6. Mannik, M., Bume, K. L., Clark, W. S., *ibid.*, 1958, v1, 410.
7. Singer, J., *Proc. 1st Conf. on Serological Reactions of Rheumatoid Arthritis, Arthritis and Rheumatism Fn.* 1957, 60.

Received June 12, 1959. P.S.E.B.M., 1959, v102.

Studies of Properdin System in Human Cancer Cell Cultures.* (25144)

CHESTER M. SOUTHAM, MAY TAI AND ELIAS L. GREENE
Sloan-Kettering Inst. for Cancer Research, N. Y. City

The demonstration of low serum properdin titers in many patients with advanced neoplastic diseases(1-5) and the correlation of this phenomenon with diminished ability to reject homotransplants of cultivated human cancer cells(1,6) impels direct study of the effects of properdin on growth of neoplasms. The present paper reports failure of properdin to affect growth of human cancer cells in tissue culture, and the effects of incubation in tissue cultures on complement components and properdin.

Methods. Properdin (P) was prepared from normal human serum by 2 methods: adsorption onto zymosan (Z) at 17°C and subsequent elution of P from resulting P-Z complex(10), or a modification of the Cohn serum fractionation technics utilizing ethanol precipitation(16). These preparations were supplied by Dr. Louis Pillemer of Western Reserve University and Dr. Benjamin Sanders of Merck, Sharp and Dohme Labs respectively. Human serum reagents lacking P (RP) or lacking P and C'3 (R3) but containing all other components of the properdin system were prepared by adsorption with zymosan at 17°C and 37°C respectively(9,10). In studies of P prepared by Z adsorption, the same serum was the source of both P and RP. All preparations of P, RP, and R3 were tested in tissue cultures singly (*i.e.* in absence of complete P system) to eliminate preparations with innate cytoxicity. They were stored at −10°C or −60°C and thawed at room temperature immediately before use. P analyses were performed by the zymosan (hemolytic) technic of Pillemer without modification(10). All RP, R3, embryo extract, pleural fluid, and ascitic fluid preparations used had no titratable P (<1. U/ml). Parallel C'3 titrations on RP preparations in presence and absence of Z suggest that residual P was less than 0.5 U/ml. Since the tissue-culture media contained not more than 50% serum products, it follows that media without added P had residual P levels of <0.5 U/ml and probably <0.25 U/ml. For convenience these media are referred to as properdin-free. C' and C' components were assayed by the technic of Ecker, Pillemer and Seifter(17) except that 50% hemolysis end point was used instead of 100%. The hydrazine method was used for preparation of the R4. Percent hemolysis was estimated by visual comparison with standards prepared from same erythrocyte suspension used for the test. All P assays and all C' and C' component assays from a single experiment were done simultaneously using the same reagents throughout. At least one normal human serum standard (and in properdin studies, a purified properdin standard) were included in all titrations to check

* These studies supported by research grants from Nat. Cancer Inst., U.S.P.H.S., and Phoebe Waterman Fund. The authors are also greatly indebted to the late Dr. Louis Pillemer and his staff, without whose cooperation and assistance these studies could not have been initiated.

the reagent systems. All specimens were stored in mechanical deep freeze (–60° to 70°C) from time of collection to time of titration. Two human cancer cell lines, Toolan's HEp #2(7) and Osgood's J-111(8), were cultivated for approximately 12 months prior to these experiments in properdin-free tissue culture media consisting of 45% Gey's salt solution, 5% chick embryo or beef embryo extract, and 50% ascitic or thoracic fluids from cancer patients. Subcultures were then planted in tubes and fed with 1 ml of another properdin-free medium consisting of 45% Gey's or Eagle's solution, 5% chick embryo extract, and 50% properdin-free serum (RP or a mixture of RP and R3). Gey's solution (11) was used for HEp #2 cells, and Eagle's solution(12) for J-111 cells. After cultivation in these media for 1-3 weeks to allow adaptation of cells to the RP serum, P (in solution in Gey's or Eagle's solution) was added to some to produce the desired final concentrations of P. Cultures were incubated at 37°C in stationary tubes without plasma clot and were refed with the appropriate media at intervals of 1 to 7 days. Representative samples of the old media which were removed when refeeding were titered for residual properdin and complement. Maximum pH variation in the cultures was 6.5 to 7.5. Magnesium and calcium ion concentration in the various media were calculated to be in the magnitude of 1×10^{-3} and 2×10^{-3} molar respectively. In studies of homotransplantability (Exp. 1), cultures were refed every 4-7 days, and after observation for 3 weeks or more, tubes containing identical nutrients were trypsinized, pooled, and transferred to milk dilution bottles for further cultivation in 10 ml of same media. Finally, they were harvested by trypsinization, washed twice, resuspended in Gey's salt solution, counted by hemocytometer, and transplanted subcutaneously to volunteer patients with advanced cancer and low serum properdin titers. Implant sites were observed daily for evidences of growth of homotransplanted cells and after nodules appeared they were measured daily until excised simultaneously for comparative microscopic study. In studies involving serial titration of C′ and P (Exp. 3), cultures and blanks were in milk-dilution bottles containing 12 ml of medium. For this study the medium was 35% RP, 15% R3, 5% chick embryo extract, and 50% Gey's solution or properdin in Gey's solution. Aliquots of 2 ml were removed from each bottle (and not replenished) at each of the 4 time intervals.

Results. Exp. 1. Prolonged exposure to properdin followed by homotransplantation. HEp 2 and J-111 cells were cultivated in media containing 0, 5, or 20 U of P/ml for 6 weeks with replenishment of medium every 5 days. There was no effect on cell morphology or multiplication rate as judged by microscopic observation of stained and unstained cultures. Total cell counts at end of cultivation period indicated that cells had increased between 6-fold and 30-fold without relation to P content of the medium. These differences are not significant. After each refeeding the cultures contained a complete complement system as measured by hemolytic effect of the medium on sensitized sheep erythrocytes, but complement activity did not persist much longer than 24 hours (see Exp. 3). Thus, these cells were only intermittently exposed to a complete P′ system. P levels in these cultures fell 50% or less during 5 days of incubation. Homotransplantation of these HEp #2 cells into a volunteer patient with advanced malignant melanoma and serum P level of <1 U/ml resulted in growth of a nodule at each implant site, with no significant differences in growth rate or final nodule diameters. Biopsies of all 4 nodules on day 14 showed identical histologic appearance with good growth of the implanted cells and no inflammatory or other regressive changes. Homotransplantation of the J-111 cells into another melanoma patient with a serum properdin titer of <1 U/ml produced only a vaguely palpable thickening at implantation sites by the 27th day after inoculation when the patient died. At autopsy the only J-111 nodule which could be located for excision was that produced by cells which had been grown in a properdin concentration of 20 U/ml. In histologic sections there were sheets of healthy appearing cells of the implanted type and no inflammatory or regressive change. Although this reimplantation

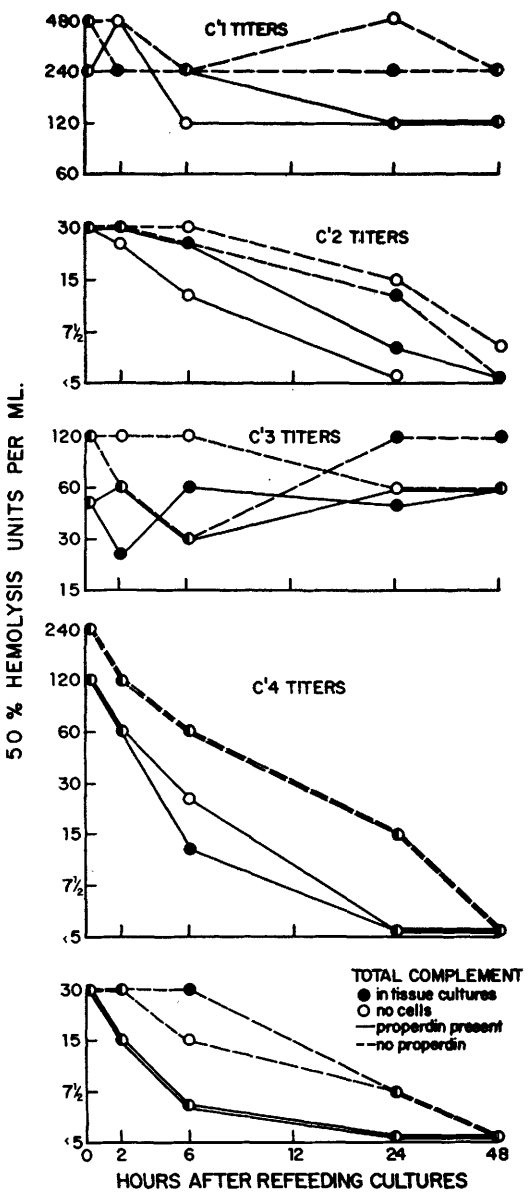

FIG. 1. Total complement and complement component titers in tissue culture media in Exp. 3 (see text). Black dots (●) indicate media from HEp 2 cell tissue cultures. Open circles (○) indicate media from "blank" culture bottles, *i.e.*, no cells present. Solid lines indicate media containing properdin (20 U/ml without change for duration of experiment). Dotted lines indicate properdin-free media. Titers and rate of fall of C'1, C'2, C'4, and total complement were not significantly affected by cells, but in presence of properdin (with or without cells) titers were lower. Titers of C'3 varied erratically during first 6 hr in presence of properdin or cells but no significant changes persisted after 24 hr.

study of J-111 cells did not permit adequate comparison between the 4 preparations, it is clear that cell growth potential was not abolished by cultivation in the presence of properdin. In other cancer patients homotransplantation studies with J-111 cells grown in properdin-free media gave similar nodule growth and microscopic appearance.

Exp. 2. Cultures continuously exposed to a complete properdin system. Because serial complement titrations of tissue culture media indicated that little complement activity remained after 24 hours of incubation, cultures of HEp 2 cells were refed every 24 hours for 6 days. Duplicate cultures were grown in each of 5 media as follows: Complete P system (RP & R3 & P); P but no C′ (Heated RP & R3); C′ but no P (P heated); C′ but no P (P omitted); neither C′ nor P (RP & R3 heated, P omitted). Properdin when present titered 8 U/ml. Total C′ when present titered 40 U/ml initially and approximately 5 U/ml after 24 hours incubation. Heated preparations contained no detectable P or C′. All cultures grew well throughout the study without relation to presence or absence of C′ or P or both, as judged by daily microscopic observation of living cultures.

Exp. 3. Behavior of complement components and properdin in tissue cultures. To study rate of disappearance of C′ components and P, tissue culture medium containing a complete C′ system was incubated in bottles with and without growing HEp 2 cells and with and without P. Aliquots were removed after incubating for 2, 6, 24, and 48 hours. P showed no significant change. Total complement activity in bottles containing P fell rapidly during the first 6 hours of incubation and disappeared (<5 U/ml) by 24 hours. In bottles containing no P′ the fall-off of C′ activity was significantly slower, but C′ was undetectable by 48 hours (Fig. 1).

C′ 2 titers essentially duplicated the total C′ titer and thus C′2 appeared to be the limiting component at all times (Fig. 1). C′4 was not a limiting component initially but fell to levels nearly as low as C′2 by 6 hours. It dropped more rapidly in presence of P. C′1 was never a limiting factor (Fig. 1). In the presence of P, C′1 titer fell more rap-

idly than in absence of P, but it did not drop further after 24 hours. No drop of C'1 was demonstrated in bottles lacking P. Since C'1 levels were high and since titrations were based on dilutions in geometric progression, an absolute change in C' titers equal to that observed for C'2 would not have been detected (*e.g.* a decrease of C'1 from 240 U/ml to 200 U/ml would not be detectable by this technic, whereas an equal drop in C'2 would reduce it from normal levels to zero).

C'3 was never a limiting factor. It showed erratic fluctuations at 2 and 6 hours in bottles containing P or cells or both, but there were no significant differences at 24 or 48 hours.

As in all other studies, the cells grew well in all tubes throughout experiment.

Discussion. Under conditions of this study human properdin at physiologic concentrations had no cytotoxic effect. It follows that if serum properdin plays any part in the process of homo- and hetero-transplant rejection, a possibility suggested by several studies with human and animal systems(1,13,14,15), it must either require combined action with additional factors for its anticellular effect, or must act indirectly. The tissue culture media which were used contained all factors presently known to be necessary for reactivity of properdin in the zymosan assay (calcium and magnesium ions and the 4 components of complement). However, there is no reason to assume that these are the only components involved in other systems in which properdin may function. On the other hand, there is nothing in the experiments cited above which proves that properdin participates in homotransplant rejection. All observations could be equally well interpreted as indicating merely that properdin levels vary in parallel with some as yet undefined factors which *are* directly involved in rejection of transplanted cells.

The possibility that concentrations of properdin greater than 20 U/ml might have cytotoxic effects was not investigated, but seems irrelevant to the purpose of this investigation, since a concentration of 20 U/ml is well above the maximum normal level in human serum.

It is recognized that these cultures were not grown in complete absence of P, but merely at P concentrations below ½ U/ml. While it is conceivable that if cells were grown in complete absence of P, they might be adversely affected by addition of P, this possibility seems to have little application to the problem under study since in the previous clinical studies(1,6) homotransplant rejection was impaired when serum P levels were 2 U/ml or lower.

The fact that after a week of incubation in tissue culture 50% or more of original properdin content was still detectable, indicates that the cells were in contact with properdin throughout the entire study and that properdin is not rapidly metabolized by these cells.

Failure of growing cells to affect complement titers likewise indicates that complement is not rapidly metabolized by the cells. It also suggests that no reaction occurred between P and the cells, since it might be expected (by analogy with other properdin reactions) that if such a reaction occurred, complement would have participated in the reaction.

The rapid fall of C'2 and C'4 titers during incubation of the tissue culture media might be attributable to chemical lability without assuming any serologic reaction, but the fact that these titers fell even more rapidly when properdin was present, and that C'1 also fell in the presence of P, suggests that these 3 complement components may react with P even in the absence of a properdin-binding substrate. (The possibility of a P-binding substance in serum reagents is not excluded, but seems unlikely since it should have reacted *in vivo* or during the incubation steps in preparation of RP or R3.)

The erratic, but transient falls in C'3 titers in tissue culture media are unexplained. They suggest the possibility that C'3 may have reacted with properdin or with cells to form reversible complexes which made the C'3 temporarily inaccessible to the hemolytic detection system.

Conclusions. 1) Neither properdin (up to 20 U/ml) nor absence of detectable properdin (<½ U/ml) had any apparent influence upon growth rate or morphology of human cancer cells HEp #2 or J-111 in tissue culture, nor upon their ability to propagate on subsequent

homotransplantation. 2) Neither properdin nor complement was rapidly metabolized or inactivated by these cells in tissue culture. 3) Addition of properdin to tissue culture medium (in presence or absence of cells) caused a fall in C'1 titer and accelerated the fall of C'2 and C'4.

1. Southam, C. M., Pillemer, L., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 596.

2. Rottino, A., Levy, A., Conte, A., *Cancer,* 1958, v11, 351.

3. Isliker, H. C., *Verhandlunger der Deutsch Gesellschaft fur innere Med.,* 1956, v62, 197 (and personal communication).

4. Eyquem, A., *Sixth Congress of European Hematol. Soc.,* Copenhagen, 1957.

5. Soulier, J. P., Menache, D., *Acta Haematol.,* 1958, v20, 260.

6. Southam, C. M., Moore, A. E., Rhoads, C. P., *Science,* 1957, v125, 158.

7. Moore, A. E., Sabachewsky, L., Toolan, H. W., *Cancer Research,* 1955, v15, 598.

8. Osgood, E. E., Brooke, J. H., *Blood,* 1955, v10, 1010.

9. Pillemer, L., Blum, L., Lepow, I., *Science,* 1954, v120, 279.

10. Pillemer, L., Blum, L., Lepow, I., Wurz, L., Todd, E. W., *J. Exp. Med.,* 1956, v103, 1.

11. Gey, G. O., *Am. J. Cancer,* 1936, v27, 45.

12. Eagle, H. J., *J. Exp. Med.,* 1955, v102, 102.

13. Palm, J. E., *Proc. Am. Assn. Cancer Res.,* 1956, v2, 138 (abst.).

14. Herbut, P. E., Kraemer, W. H., *Cancer Research,* 1956, v16, 408.

15. Bradner, W. T., Clarke, D. A., Stock, C. C., *Proc. Am. Assn. Can. Res.,* 1957, v2, (abst).

16. Spicer, D. S., Priester, L. I., Smith, E. V. C., Sanders, B. C., *J. Biol. Chem.,* 1959, v234, 838.

17. Ecker, E. E., Pillemer, L., Seifter, S., *J. Immunol.,* 1943, v47, 181.

Received June 1, 1959. P.S.E.B.M., 1959, v102.

Comparison in Rabbits of Hypoglycemia and B Cell Degranulation After Various Sulfonylureas.* (25145)

SYDNEY S. LAZARUS AND BRUNO W. VOLK

Isaac Albert Research Inst. of Jewish Chronic Disease Hospital, Brooklyn, and Dept. of Pathology, Albert Einstein College of Medicine, Bronx, N. Y.

It has been shown unequivocally that sulfonylureas produce degranulation of the pancreatic B cells of rats, rabbits and dogs when administered over prolonged periods(1-3). This degranulation has been interpreted(2) as indicative of suppression of insulinogenesis similar to that observed during chronic hypoglycemia induced by insulin administration. However, we demonstrated previously(4) that in rabbits insulin induced hypoglycemia did not produce degranulation, whereas hypoglycemia of equivalent depths and duration produced by tolbutamide caused varying degrees and frequently complete degranulation of the B cells. We undertook to determine whether the hypoglycemic sulfonylureas would produce pancreatic B cell degranulation even though their hypoglycemic action was prevented by simultaneous infusion of glucose. Furthermore, since the hypoglycemic effectiveness of these drugs apparently depends on their ability to increase insulin output from B cells and since this increased insulin output will be reflected in the extent of B cell degranulation, we attempted to correlate their blood sugar lowering efficacy with degree of B cell degranulation.

Material and methods. We used 72 New Zealand white rabbits of either sex, weighing 2.5 to 4 kg divided into 3 main groups. 1) *Single injections.* Eighteen normal rabbits, after overnight fast, each received intravenously 125 mg/kg of tolbutamide or chlorpropamide, or metahexamide. Furthermore, 6 animals received 1 cc/kg of saline intravenously and served as controls. Blood was withdrawn from marginal ear vein immediately before and at hourly intervals for 5 consecutive hours after administration of each test dose. 2) *Multiple injections.* Eighteen

* Supported by grant from Upjohn Co., Kalamazoo, Mich.